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14. ABSTRACT Traumatic Brain Injury (TBI) is a well-established inducer of temporal lobe epilepsy (TLE), a frequently medically intractable and permanent epilepsy syndrome. Unlike many TLE models, which cause global brain injury that do not replicate the human condition, or other TBI models, which do not induce TLE reliably, the controlled cortical impact (CCI) model of posttraumatic epilepsy in mice results in localized cell loss, synaptic reorganization, and development of TLE. Abnormalities in inhibitory neurotransmission are important aspects of TLE in several animal models. Under this award, the CCI model was established in all three collaborating laboratories. Specific parameters of injury associated with epileptogenesis were determined. It was determined that upregulation of the JaK/STAT3 pathway in the injured hippocampus occurs after CCI, which could be blocked by post-injury administration of a JaK/STAT3 inhibitor. Blocking JaK/STAT3 activity did not prevent loss of GABA cells in the injured hippocampus. Inhibitory postsynaptic currents in the dentate gyrus ipsilateral to the injury were reduced in frequency weeks after the injury, recapitulating findings in other models in which aspects of epileptogenesis were attenuated by STAT3 inhibition. These results critically establish model parameters and control measurements, and provide the basis for remaining proposed experiments.					
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INTRODUCTION:

This research addresses the FY10 PRMRP topic area of Epilepsy. Traumatic Brain Injury (TBI) is a well-established etiology of temporal lobe epilepsy (TLE), a frequently medically intractable and often progressive epilepsy syndrome. Much evidence indicates that abnormalities in inhibitory neurotransmission are important in TLE. Our overall hypothesis is that Janus Kinase (JaK)/Signal Transducer and Activator of Transcription 3 (STAT3) pathway activation after TBI leads to GABA(A) receptor $\alpha 1$ subunit gene (*Gabra1*) repression and is a critical mediator of post-traumatic epileptogenesis and epilepsy progression. The JaK/STAT pathway has not been studied in post-traumatic epilepsy, but beyond its role in *Gabra1* regulation, it is known to be an important regulator of neuronal proliferation, survival and gliogenesis, all of which may be important contributors to epileptogenesis. Specifically, long-term decreases in expression of the GABA(A) Receptor $\alpha 1$ subunit gene (*Gabra1*) in the hippocampal dentate gyrus have been shown to occur in the pilocarpine-model of TLE in animals and preventing this repression has been shown to inhibit development of epilepsy in this model. In addition, decreases in expression of $\alpha 4$ subunits, which contribute to the generation of tonic GABA currents and potentially regulate granule cell activity, have also been reported in TLE and after TBI. It has been recently established that transcriptional repression of GABA receptor subunits in the pilocarpine-model of TLE is mediated by inducible cAMP early repressor and phosphorylated CREB, and that ICER transcription is driven by the JaK/STAT signaling cascade. Pharmacological inhibition of the JaK/STAT3 pathway prevents *Gabra1* repression and inhibits progression of epilepsy in the pilocarpine model. Preliminary data indicated that spontaneous seizures activate the JaK/STAT3 pathway in the pilocarpine model, suggesting this pathway may be involved in the maintenance and progression of TLE. Preliminary evidence shows that the JaK/STAT3 pathway is activated following TBI in injured hippocampus and cortex after a *diffuse* injury, and data obtained under this award indicate that phosphorylated STAT3 is increased shortly after focal injury near the injury site, with concurrent changes in $\alpha 1$ and $\alpha 4$ GABA receptor subunit expression. The impact however, on GABA(A) receptor subunit function, and whether these are important mechanisms of post-traumatic epileptogenesis are unknown. Focal injury caused by controlled cortical impact (CCI) has been shown to induce cell loss, synaptic reorganization, and TLE with spontaneous seizures in mice (Hunt et al., 2009; 2012). In order to assess changes in GABA responsiveness and alteration of those changes by Jak/STAT3 inhibition, we coordinated procedures across three labs, established that the fundamental changes in JaK/STAT3 activity occurred, and ensured that the JaK/STAT3 blocker was effective in this model. We also examined the effect of STAT3 blockade on indicators of neuronal proliferation and survival. Specific experiments on critical aspects of GABA function were performed in all years to address the Specific Aims of the study. We examined functional changes in specific GABA receptor-mediated responses in dentate granule cells from the CCI-injured mouse and the effects of blocking STAT3 phosphorylation on those changes. Results of these studies provide new information regarding the role of the JaK/STAT signaling cascade in regulation of brain inhibition and epileptogenesis after traumatic brain injury, and have the promise of leading to new therapies for modification of post-traumatic epileptogenesis.

BODY:

Aim 2: Performed in laboratory of Dr. Bret N. Smith at University of Kentucky

Determine whether activation of the JaK/STAT pathway and downregulation of *Gabra1* transcription following TBI result in altered inhibitory synaptic neurotransmission in the hippocampus that may contribute to epileptogenesis.

Task 1: Determine whether benzodiazepine modulation of IPSCs in dentate granule cells (DGCs) is altered after CCI and whether this alteration is prevented by inhibiting STAT3 phosphorylation with WP1066. (Timeframe months 1-18).

Task 1a. Induce TBI using CCI model in adult CD-1 mice (200 mice used, 20 sham-injured controls, 80 injured untreated, 20 sham-injured, WP1066-treated controls, 80 injured WP1066-treated; Timeframe months 1-18).

Status: Complete

1. Verified parameters of CCI injury that result in epileptogenesis and markers of the development of epileptic phenotype after 8-12 weeks post-injury. Approximately 50 mice were injured with CCI in the last year, 20 of which were injected with WP-1066. Based on collaborators' findings that more severe injury results in greater STAT3 phosphorylation, studies included a few mice with greater injury.
 - a. The spatial extent of moderate and severe injury-related epileptogenic changes with respect to distance from impact point was determined.
2. Establish that phosphorylation of STAT3 is upregulated and was inhibited by WP1066 in this model.
 - a. Establish effect and localization of STAT3 phosphorylation after CCI.
 - b. Establish effect of WP1066 on STAT3 phosphorylation after CCI.
 - c. Based on collaborators preliminary results, initiated studies to examine the extent of STAT3 phosphorylation after more severe injury.

Accomplishments:

1. Determined precise parameters of effective CCI (1 mm depth) to obtain epileptogenic phenotype. Phenotype changes were assessed for more severe injury (2 mm), based on preliminary findings from collaborators. In our hands, 2 mm injury essentially destroyed the hippocampus and dentate gyrus and was determined to be too severe to study effects of injury ipsilaterally. With intermediate injury (1.5, 1.2 mm), damage was either similar to 2 mm (i.e. 1.5 mm) or to 1 mm (i.e., 1.2 mm). The 1 mm injury was therefore used for all studies.
2. Completed determination that CCI increased STAT3 phosphorylation ipsilateral to the injury after 24 hours, but not contralaterally. Western blot analyses of hippocampi from CCI-injured and control mice were concluded after 1 mm depth injury. Both STAT and phosphorylated STAT (pSTAT) protein expression were compared semi-quantitatively 24 hr after injury (**Fig 1**). Comparisons were made for hippocampi ipsilateral to the injury, contralateral to the injury, and in sham-operated controls. STAT and pSTAT levels were normalized to those for β -actin. Results indicated that pSTAT ($p < 0.05$), but not STAT ($p > 0.05$), expression was increased in the hippocampus ipsilateral to the injury. We further determined that treatment at 30 and 90 min after CCI (1 mm) with WP1066 (50 mg/kg) inhibits STAT3 phosphorylation in mice. Full analysis was reported (Butler et al., 2012; Boychuk et al., 2012).

Conclusion: The biochemical reaction required to perform further analyses of GABA currents is evident after CCI ipsilateral to the 1 mm injury, but not contralaterally. This means that the contralateral dentate gyrus can serve as a control for electrophysiological analyses.
3. Completed analysis of histopathological features (i.e., MFS and hilar GABA neuron loss) in the dentate gyrus consistent with epileptogenesis (**Figs. 2, 6**). Mossy fiber sprouting analysis was completed in year one (Hunt et al., 2012). Assessment of hilar inhibitory neuron loss was completed in year 2; preliminary

data were included in the previous progress report (Butler et al., 2012; Boychuk et al., 2012). The distribution of inhibitory neuron loss was compared to previous results on mossy fiber sprouting. Significant GABA neuron loss was limited to the injury epicenter and an area extending 800 μ m temporal (ventral) to the injury; areas septal (dorsal) to the injury were unaffected, similar to the distribution of mossy fiber sprouting. Contralaterally and at more ventral levels ipsilaterally, hippocampal pathology was not observed (Butler et al., 2012). We further determined that treatment with WP1066 did not affect GABA neuron loss. Full analysis of mice from the two injection paradigm (50 mg/kg each), delivered at 30 and 90 min post-injury (1 mm) was completed. The WP1066 treatment did not, however, alter the degree of GABAergic hilar interneuron cell loss ipsilateral to the injury (Butler et al., 2012). Conclusions: The histopathology most likely to be required for studying changes in GABA currents after CCI was associated with hippocampal evulsion, which was observed when using beveled tips (versus rounded tips) to a depth of 1 mm. Treatment with WP1066 showed no evidence of influence on cell proliferation, axon growth, or neuronal survival.

Supporting Data: **Figure 1.** Western blot results indicating increased STAT3 phosphorylation after 1 mm CCI ipsilateral to the injury and inhibition of pSTAT3 by systemic WP1066 treatment (50 mg/kg, i.p.) at 30 and 90 min after CCI injury. **Figure 2.** The hilar GABA neuron loss after CCI and the lack of effect of WP1066 treatment on hilar neuron loss. These data were completed in year 2, but reported in revised progress report for year 1.

Task 1b. Measure effects of zolpidem on IPSCs in DGCs from WP1066-treated and untreated control mice and in mice shortly (i.e., 1-6 weeks) after CCI injury. (100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 1a; Timeframe months 1-9).

Status: Completed

Accomplishments:

1. IPSCs were recorded from granule cells from sham control, CCI-injured, and CCI-injured+WP1066 treatment mice (1-6 weeks post-injury; 8-12 mice per group). IPSC frequency, amplitude, and decay time constants were measured. Analysis is complete for amplitude and frequency; analysis is being verified for decay time constant and that process will be complete within 30 days.
2. Applied α 1 subunit-selective benzodiazepine agonist, zolpidem, and measured IPSC frequency, amplitude and decay time constant in neurons from sham control, CCI-injured, and CCI-injured+WP1066 treatment mice. Analysis is complete for effect of zolpidem on amplitude and frequency; analysis is being verified for effect on decay time constant and that process will be complete within 30 days: benzodiazepine agonist tends to increase IPSC frequency and time constant, and these effects were not statistically different in WP1066-treated mice.

Supporting Data: **Figure 3.** Graphical description of results from IPSC analysis within 10-21 days after injury.

Task 1c. Measure effects of zolpidem on IPSCs in DGCs from WP1066-treated and untreated control mice and in mice 6-10 weeks after CCI injury. (100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 1a; Timeframe months 4-18)

Status: Completed

Accomplishments:

1. IPSCs were recorded from granule cells from sham control, CCI-injured, and CCI-injured+WP1066 treatment mice (1-6 weeks post-injury; 8-11 mice per group). IPSC frequency, amplitude, and decay

time constants were measured: benzodiazepine agonist tends to increase IPSC frequency and time constant, and these effects were not statistically different in WP1066-treated mice.

2. Applied $\alpha 1$ -subunit selective benzodiazepine agonist and measured IPSC frequency, amplitude and decay time constant in neurons from sham control, CCI-injured, and CCI-injured+WP1066 treatment mice. Analysis is complete for effect of zolpidem on amplitude, frequency, and decay time constant: benzodiazepine agonist tends to increase IPSC frequency and time constant, and these effects were not statistically different in WP1066-treated mice.

3. Tonic GABA currents were minimally affected by zolpidem in neurons from control mice. However, at both 1-6 weeks and 10-13 weeks, zolpidem induced a significantly larger tonic current in neurons from CCI-injured mice ($p < 0.05$). In neurons from CCI-injured mice treated with WP1066, zolpidem application resulted in a small Itonic that was similar to that observed in control mice and significantly less than that observed in untreated, CCI-injured mice. This effect was observed at both the 1-6 week and 10-18 week timepoints.

Supporting Data: **Figure 4.** Graphical description of results from IPSC analysis 6-10 weeks after injury.

Figure 5. Graphical depiction of effects of zolpidem on Itonic at early and late time points. See also

Table 1 below for analysis of IPSC amplitude, frequency, and time constant for control, CCI-injured, and CCI-injured+WP1066 treated mice.

Task 1d. Perform Timm histological analysis, to detect mossy fiber sprouting in all slices from which recordings are made. (200 mice needed; same mice as in Tasks 1a-c; Timeframe months 1-18).

Status: Completed

Accomplishments:

1. Approximately 100 mice were treated with CCI, 50 of which were treated with WP1066; 80 controls were used.
2. Timm staining did not reveal mossy fiber sprouting in any group at the early time-point, as expected.
3. At 6-10 weeks post-injury, Timm staining was observed in CCI-injured, but not sham control mice. CCI-injured+WP1066 treated mice displayed Timm distribution similar to CCI-injured mice at 6-10 weeks post-injury.

Supporting Data: See **Figure 6.** Timm scores for the three groups analyzed: Control = 0.13 ± 0.09 ($n=16$); CCI-injured = 1.93 ± 0.30 ($n=16$; $p < 0.05$ vs control); CCI-injured+WP1066 = 1.88 ± 0.30 ; ($n=16$; $p < 0.05$ versus control; $p > 0.05$ versus CCI-injured; ANOVA). Mossy fiber sprouting was not reduced by WP1066 treatment after CCI injury.

Task 2: Determine if furosemide modulation of IPSCs in DGCs is altered after CCI and if inhibiting STAT3 phosphorylation with WP1066 prevents the alteration. (Timeframe: months 19-36)

Task 2a. Induce TBI using CCI model in adult CD-1 mice (200 mice used, 20 sham-injured controls, 80 injured untreated, 20 sham-injured, WP1066-treated controls, 80 injured WP1066-treated; Timeframe months 19-36).

Status: Completed

Accomplishments: Accomplishments identical to Task 1, 1a.

Supporting Data: Same as Task 1, 1a.

Task 2b. Measure effects of furosemide on IPSCs in DGCs from WP1066-treated and untreated control mice and in mice 1-6 weeks after CCI injury. (100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 2a; Timeframe months 19-27).

Status: Completed.

Accomplishments: Recording experiments were performed in DGCs from 7 mice in each group. Furosemide application decreased IPSC frequency, but not amplitude, in CCI-injured mice relative to control. However, it was determined that furosemide also had non-specific effects on regulators of ionic currents in addition to those mediated by $\alpha 4$ -subunit containing GABA receptors, including energy-dependent Cl^- co-transporters. These results were therefore inconclusive.

Supporting Data: none

Task 2c. Measure effects of furosemide on IPSCs in DGCs from WP1066-treated and untreated control mice and in mice 6-10 weeks after CCI injury (months 4-18). 100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 2a; Timeframe months 19-36).

Status: Completed

Accomplishments: Recording experiments were performed in DGCs from 5 mice in each group. Furosemide application decreased IPSC frequency, but not amplitude, in CCI-injured mice relative to control. However, it was determined that furosemide also had non-specific effects on several currents in addition to those mediated by $\alpha 4$ -subunit containing GABA receptors, including energy-dependent Cl^- co-transporters. Results from these experiments were therefore inconclusive.

Supporting Data: none.

Task 2d. Perform Timm histological analysis, to detect mossy fiber sprouting in all slices from which recordings are made. (200 mice needed; same mice as in Tasks 2a-c; Timeframe months 19-36).

Status: Completed

Accomplishments: Accomplishments identical to Task 1, 1d.

Supporting Data: Same as Task 1, 1d.

Task 3: Determine if THIP-induced tonic GABA currents in DGCs are altered after CCI and if the alteration is prevented by inhibiting STAT3 phosphorylation with WP1066. (Timeframe months 10-27)

Task 3a. Induce TBI using CCI model in adult CD-1 mice (100 mice needed, 30 sham-injured controls, 30 injured untreated, 10 sham-injured, WP1066-treated controls, 30 injured WP1066-treated; Timeframe months 10-27)

Status: Completed

Accomplishments: Accomplishments identical to Task 1, 1a.

Supporting Data: Same as Task 1, 1a.

Task 3b. Measure THIP-induced tonic GABA current in DGCs from WP1066-treated and untreated control mice and in mice 1-6 weeks after CCI injury. (100 mice needed, 30 sham-injured controls, 30 injured untreated, 10 sham-injured, WP1066-treated controls, 30 injured WP1066-treated; subset of mice in Task 3a; Timeframe months 10-18).

Status: Completed

Accomplishments: 4,5,6,7-Tetrahydroisoxazolo[5,4c]pyridine-3-ol hydrochloride (THIP) currents were measured in neurons from control, CCI-treated, and CCI-injured+WP1066 treatment mice (7-11 mice per group) at 1-6 weeks post injury.

Supporting Data: **Figure 7.** THIP-induced tonic GABA currents are reduced in neurons from CCI-injured mice; WP1066 treatment did not affect the THIP-induced current amplitude at 1-6 weeks post-injury.

Table 1. THIP application decreased IPSC frequency in all groups to a similar extent. The effect was similar for animals examined at 1-6 weeks and 10-18 weeks. WP1066 had no effect on the THIP effect on IPSCs.

Task 3c. Measure THIP-induced tonic GABA current in DGCs from WP1066-treated and untreated control mice and in mice 6-10 weeks after CCI injury (months 4-18). 100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 3a; Timeframe months 19-27).

Status: Completed

Accomplishments:

1. Determined that THIP-induced tonic GABA current in DGCs 6-10 weeks post-injury are reduced in amplitude relative to sham-operated controls and contralateral DGCs (n=7-14 cells in 7 mice from each group; $p < 0.05$). Based on preliminary results from collaborators in Colorado and on data published recently elsewhere, these experiments were initiated to identify potential functional changes due to altered $\alpha 1$ vs $\alpha 4/\delta$ subunit-containing GABA receptor expression weeks after injury, corresponding to time points where epilepsy is established in this model (i.e., 6-10 weeks post-injury). Granule cells were recorded in ex vivo slices taken from control mice and from slices taken contralateral and ipsilateral to injury site in CCI-injured mice, 6-10 weeks post-injury. Cells were voltage-clamped at 0 mV and THIP (3 μ M) was bath-applied to induce an outward current, ostensibly due to activation of δ subunit-containing GABA receptors (most likely $\alpha 4/\delta$). Bicuculline methiodide (30 μ M) was applied to block all GABA receptors and determine the total available tonic GABA current. Conclusions: Total tonic GABA current in granule cells from control versus CCI-injured mice. THIP-current in cells contralateral to the injury are not different from those in control mice. However, THIP-induced tonic current is reduced by about 50% ipsilateral to the injury in cells from CCI-treated versus controls ($p < 0.05$) or in cells contralateral ($p < 0.05$) to the injury.
2. Recordings were made from 12 granule cells in 7 WP1066-treated CCI-injured animals and tonic GABA and THIP currents were compared to results from control mice and CCI-injured mice. As in other groups, tonic GABA current was unaffected in WP1066-treated CCI-injured mice ($p > 0.05$), as expected. The THIP-current was significantly reduced by about 50% ipsilateral to the injury in cells from WP1066+CCI-treated versus controls or in cells contralateral to the injury ($p < 0.05$). The THIP current in the WP1066+CCI-injured group was not different from CCI-treated group ($p > 0.05$).

Supporting Data: **Figure 7.** THIP-induced changes in tonic GABA current in sham-operated controls, CCI-injured, and CCI-injured+WP1066 after 6-10 weeks post injury.

Task 3d. Perform Timm histological analysis, to detect mossy fiber sprouting in all slices from which recordings are made. (200 mice needed; same mice as in Tasks 3a-c; Timeframe months 10-27).

Status: in progress

Accomplishments: Accomplishments identical to Task 1, 1d.

Supporting Data: Same as Task 1, 1d.

KEY RESEARCH ACCOMPLISHMENTS:

- Refined precise injury parameters for CCI in mice that yield consistent and reliable outcome measures. All tasks required the CCI model to be established. Determined that 1 mm injury was necessary and sufficient to induce cellular and behavioral changes associated with epileptogenesis.
- Determined that 1 mm injury causes sufficient damage to induce pSTAT3 production to initiate hypothesized signal cascade necessary to result in epileptogenic phenotype.
- Completed training of all lab personnel on project.
- Phosphorylated STAT3 levels were increased in the hippocampus ipsilateral to the injury 24 hours after CCI. By one week, there was no appreciable continued activation.
- In mice, 30 and 90 minute post-treatment of WP1066 inhibits phosphorylation of JAK/STAT3 in injured hippocampus 24 hours after CCI to control levels. All tasks required controls to demonstrate effectiveness of WP1066.
- In mouse, mossy fiber sprouting in the inner molecular layer is regionally and locally enhanced after CCI in a semi-quantitatively measurable manner. JAK/STAT3 inhibition did not affect mossy fiber sprouting 6-10 weeks post-injury.
- Selective hilar GABA interneuron loss was documented near the injury site after CCI-injury. JAK/STAT3 inhibition did not alter the cell loss.
- Total tonic GABA currents were not significantly altered in granule cells of the dentate gyrus ipsilateral to the injury 6-10 weeks after CCI in mice. However, THIP-induced tonic currents were reduced ipsilateral to the injury by 6-10 weeks after CCI-injury. Inhibition of STAT3 with WP1066 at the time of CCI-injury did not reinstate the THIP-activated tonic current ipsilateral to the injury.
- Zolpidem induced a significant increase in IPSC frequency in neurons from all groups at both the 1-6 week and 10 week time points. Inhibition of STAT3 with WP1066 at the time of CCI-injury did not alter the effect of zolpidem on IPSCs. The effect was likely due to network interactions among GABAergic inhibitory neurons.
- Zolpidem induced a significant increase in tonic current in neurons from CCI-injured mice compared to controls at both the 1-6 week and 10 week time points.
- Inhibition of JAK/STAT3 with WP1066 at the time of CCI-injury abrogated the effect of zolpidem on tonic currents.

REPORTABLE OUTCOMES: manuscripts, abstracts, presentations

1. Hunt, R.F., Haselhorst, L.A., Schoch, K.M., Bach, E.C., Rios-Pilier, J., Scheff, S.W., Saatman, K.E., and Smith, B.N. (2012) Posttraumatic mossy fiber sprouting is related to the degree of cortical damage in three mouse strains. *Epilepsy Res.* 99:167-170.
2. Butler CR, Boychuk, JA, Raible, DJ, Frey, L, Brooks-Kayal, AR, and Smith BN (2012) JAK/STAT Activation and GABA Neuron Loss After Focal Traumatic Brain Injury in Mice. *Soc. Neurosci. Abs.*, 38:247.03
3. Boychuk JA, Butler, CR, Raible, DJ, Frey, L, Brooks-Kayal, AR, and Smith BN (2012) Focal traumatic brain damage results in localized GABA neuron loss and JAK/STAT activation early following injury. *Epilepsy Curr.* 13: 53:2.007.
4. D Raible, L Frey, J Boychuk, C Butler, H Grabenstatter Y Cruz Del Angel, S Russek, B Smith and A Brooks-Kayal. JaK/STAT inhibition to prevent posttraumatic epilepsy. Poster Presentation. Rocky Mountain Regional Neuroscience Group Annual Meeting, UC AMC. 2013
5. D Raible, L Frey, J Boychuk, C Butler, H Grabenstatter Y Cruz Del Angel, S Russek, B Smith and A Brooks-Kayal. JaK/STAT inhibition to prevent posttraumatic epilepsy. Poster Presentation. CTSA national pre-doctoral meeting. 2013

6. D Raible, L Frey, J Boychuk, C Butler, H Grabenstatter Y Cruz Del Angel, S Russek, B Smith and A Brooks-Kayal. JaK/STAT inhibition to prevent posttraumatic epilepsy. Poster Presentation. Department of Neurology Research Retreat. 2013
7. D Raible, L Frey, J Boychuk, C Butler, H Grabenstatter Y Cruz Del Angel, S Russek, B Smith and A Brooks-Kayal. JaK/STAT inhibition to prevent posttraumatic epilepsy. Oral Presentation. Rocky Mountain Regional Neuroscience Group Annual Meeting. 2013
8. Butler, CB, Boychuk, JA, Frey, L, Brooks-Kayal, AR, and Smith, BN. Inhibitory signaling to dentate granule cells following traumatic brain injury. *Soc. Neurosci. Abs.*, 39: 536.08.
9. Hall, E.D., Smith, B.N., Brooks-Kayal, A., and Soltesz, I. (2014) When ski helmets aren't enough: emerging therapies for TBI and post-traumatic epilepsy. *Winter Conf. Brain Res.*

CONCLUSIONS

The CCI model was refined in mice at both University of Colorado and University of Kentucky, making future experiments feasible. Essential baseline control data was obtained to ensure that all aspects of CCI-injury and of WP1066 treatment were feasible and repeatable. Notably, 1 mm injury depth resulted in STAT phosphorylation, inhibitory cell loss, mossy fiber sprouting, and reduction in THIP-activated tonic GABA currents. A significant percentage of these animals were confirmed to express behavioral seizures. Colleagues at the University of Colorado had success with a 2 mm injury, so effects of this more severe injury on the parameters we study completed to determine if an injury severity-response relationship exists. We found that the 2 mm injury in our hands obliterated the hippocampus, making assessment of cellular changes unfeasible; 1 mm injury was used for all experiments. CCI-injury significantly phosphorylates STAT3 after injury and treatment with WP1066 significantly reduced this phosphorylation. Select GABA neuron loss is seen shortly after injury, and mossy fiber sprouting develops after several weeks post-injury, neither of which outcome was altered in WP1066-treated mice.

Total tonic GABA currents in granule cells are unaffected by the injury. However, contrary to some other models, THIP-activated tonic GABA currents are reduced in granule cells ipsilateral to the injury, suggesting a reduction in δ GABA receptor subunits (possibly $\alpha 4/\delta$ -containing), as hypothesized. Treatment with WP1066 did not reinstate the reduction in THIP-activated current, suggesting that the decrease in δ -subunit containing GABA receptors after injury is not affected by JAK/STAT3 inhibition. Previously, Raible et al (2012) showed that $\alpha 4$ -subunits are decreased one week after fluid percussion injury. Often, $\alpha 4$ -subunits pair with δ -subunits. The reduction in THIP-activated current is consistent with this finding. It is also consistent with a lack of influence of JAK/STAT3 phosphorylation on δ -subunit expression. Specific $\alpha 4$ -subunit associated effects were the target of furosemide experiments. However, since furosemide had only minor effects on tonic currents, and the drug was determined to have numerous effects not related to $\alpha 4$ -subunit modulation, results of experiments using furosemide were not conclusive. Alterations in $\alpha 1$ subunit function were targeted using zolpidem, a relatively selective benzodiazepine-like agonist. Zolpidem application resulted in a network effect on GABAergic synaptic connectivity, which was statistically enhanced in CCI-injured mice, but was not affected by treatment with JAK/STAT3 blocker. Notably, zolpidem also induced a significantly larger GABAergic tonic current in dentate granule cells from CCI-injured mice, and this effect was eliminated in neurons from mice that were treated with the JAK/STAT3 inhibitor. It is possible that CCI-injury results in increased $\alpha 1$ subunit expression selectively in granule cells, and this expression is modulated by JAK/STAT3. It is also possible that the injury induces de-novo expression of $\alpha 1, 2, 3$ or 5 subunits in granule cells. Additional experiments are ongoing to resolve this issue. A manuscript will be submitted for publication later this year, which describes GABA current changes and effects of WP1066 after CCI injury.

Summary: Novel changes in tonic GABA current amplitude were identified in CCI-injured mice. WP1066-treatment did not affect responses to THIP (i.e., $\alpha 4/\delta$ -subunit containing receptors). The increase in zolpidem-induced currents in granule cells after CCI may represent a form of compensatory regulation of tonic GABA currents, since normal granule cells do not express a significant zolpidem-induced tonic current.

Granule cell-specific upregulation of $\alpha 1,2,3$ or 5 subunits post-injury is hypothesized to contribute to this response. This unexpected result will be pursued in future experiments, since tonic currents exert a large influence on neuronal excitability. Blocking JAK/STAT3 with WP1066 immediately after injury significantly reduced the response to zolpidem post-CCI, indicating that interfering with a JAK/STAT3-initiated cascade of events after injury has profound long-term effects on granule cells that includes reducing compensatory effects on tonic currents. The function of specific forms of inhibition in granule cells requires further analysis because different GABAergic responses (i.e., synaptic IPSCs vs non-synaptic tonic current) can affect granule cell behavior differently. In general, GABA_A receptor-mediated responses are predicted to be depolarizing in granule cells, due to their relatively hyperpolarized resting membrane potential. Current carried by synaptic IPSCs seem likely to result in shunting inhibition of other, excitatory inputs. Effects on granule cell excitability of non-synaptic, tonic currents, however, are less-well characterized. The zolpidem current expressed in granule cells from CCI-injured mice could paradoxically increase excitability of the neurons by depolarizing the membrane potential. The effect of WP1066 to reduce this current might therefore be expected to reduce excitability. Additional experiments are required to assess this possibility. In WP1066-treated mice, many results are not consistent with our original overall general hypothesis that blocking JAK/STAT3 after CCI injury reduces cellular, functional, and behavioral changes associated with posttraumatic epilepsy development. However, the effect of JAK/STAT3 inhibition after CCI on zolpidem-sensitive tonic currents provides a potentially promising avenue of exploration into modulation of more specific cellular pathways to alter the progression of post-traumatic epileptogenesis.

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APPENDICES

SUPPORTING DATA:

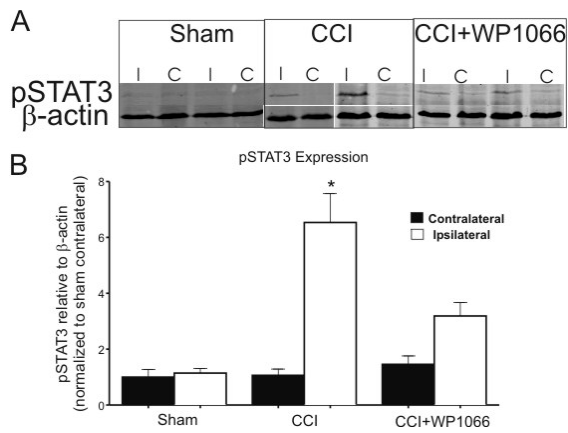


Figure 1. Western blot and representative bar graph of pSTAT3 expression relative to β -actin content in CCI-injured, sham- injured, and CCI-injured + WP1066 treatment. Expression of pSTAT3 is significantly increased in the ipsilateral hemisphere of CCI hippocampus compared to Sham 24 hr after injury. Expression of pSTAT3 in mice injected with the STAT3 inhibitor WP1066 is significantly reduced relative to CCI ($P<0.05$) and is similar to sham levels ($P>0.05$; Tukey’s ANOVA post hoc).

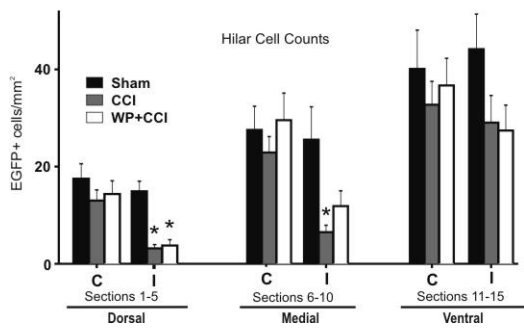


Figure 2. Hilar interneuron counts in Sham, CCI, and CCI with WP1066 treatment. Sections were taken in a 1 in 5 series at 30 μ m. Sections were divided into dorsal, medial, and ventral portions (5 section per group). Cell density in dorsal hippocampus after CCI and CCI+WP1066 treatment were significantly decreased compared to Sham in the ipsilateral hemisphere (Tukey’s ANOVA post hoc; $p<0.05$). In medial hippocampus, only CCI was significantly decreased compared to Sham ipsilateral hemisphere. There were no differences in ventral hippocampus.

There were no significant differences between groups in contralateral hemisphere or between CCI and CCI with WP1066 treatment in the ipsilateral hemisphere.

IPSCs: 10-21 days

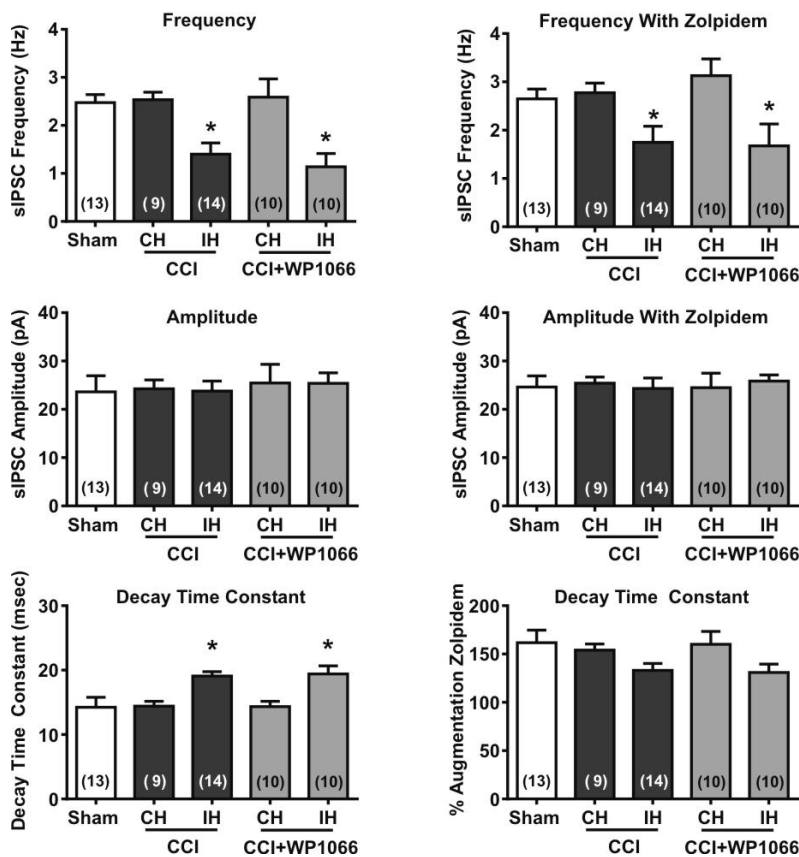


Figure 3. Graphical illustration of zolpidem effects on IPSCs 10-21 days post-injury. **Frequency** - Prior to zolpidem application, experimental groups exhibited a significant main effect of sIPSC frequency [$F(4,51) = 7.65$, $p < 0.0001$]. Multiple comparisons revealed that DGCs ipsilateral to injury from CCI or CCI+WP1066 animals had significantly lower sIPSC frequencies compared to Sham controls (Tukey's, $p = 0.013$ and $p = 0.0034$, respectively) or their contralateral cell counterparts controls (Tukey's, $p = 0.021$ and $p = 0.0027$, respectively). Ipsilateral cells from CCI versus CCI+WP1066 animals did not significantly differ in sIPSC frequency under baseline conditions (Tukey's, $p = 0.94$). A significant main effect of sIPSC frequency was also detected in the presence of zolpidem [$F(4,51) = 4.023$, $p = 0.0065$]. Multiple comparisons revealed that DGCs ipsilateral to injury from CCI or CCI+WP1066 animals had significantly lower sIPSC frequencies compared to contralateral cells from the WP1066 group (Tukey's, $p = 0.024$ and $p = 0.030$, respectively)

but not from Sham control (Tukey's, $p = 0.20$ and $p = 0.21$) or the contralateral CCI group (Tukey's, $p = 0.18$ and $p = 0.19$). **Amplitude** - No significant main effect of condition was detected between groups in the amplitude of sIPSC under baseline conditions [$F(4,51) = 0.095$, $p = 0.98$] or in the presence of zolpidem [$F(4,51) = 0.088$, $p = 0.99$]. **Decay Time Constant** - A significant main effect of condition was detected between for sIPSC decay time constant in the absence of zolpidem [$F(4,51) = 6.03$, $p = 0.0005$]. Multiple comparisons revealed that DGCs ipsilateral to injury from CCI animals possessed a significantly greater sIPSC decay time constant compared to Sham controls (Tukey's, $p = 0.011$) or their contralateral cell counterpart controls (Tukey's, $p = 0.037$). Similarly, DGCs ipsilateral to injury from CCI+WP1066 animals possessed a significantly greater sIPSC decay time constant compared to Sham controls (Tukey's, $p = 0.014$) or their contralateral cell counterpart controls (Tukey's, $p = 0.028$). A post hoc comparison of ipsilateral DGCs from CCI versus CCI+WP1066 animals detected no difference in sIPSC decay time constant (without zolpidem) for these groups (Tukey's, $p > 0.99$). In contrast, a comparison of zolpidem's percent augmentation of decay time constant revealed no significant main effects although a statistical trend was noted [$F(4,51) = 2.16$, $p = 0.087$]. A post hoc comparison of ipsilateral DGCs from CCI versus CCI+WP1066 animals detected no difference in zolpidem augmentation of sIPSC decay time constant for these groups (Tukey's, $p > 0.99$).

IPSCs: 6-10 weeks

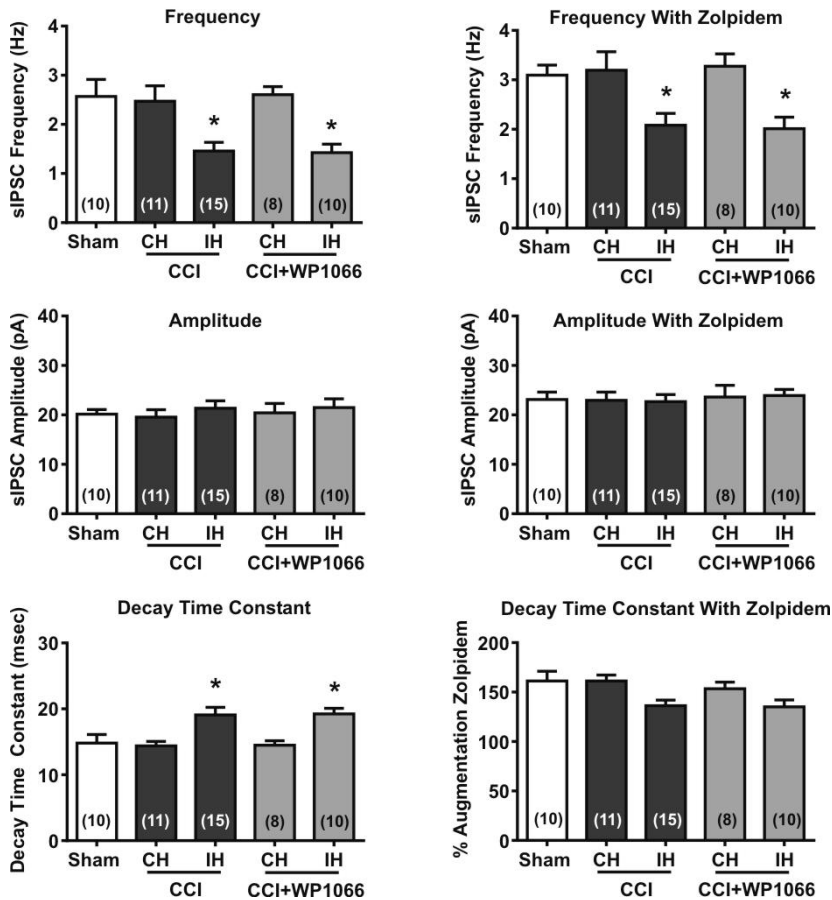


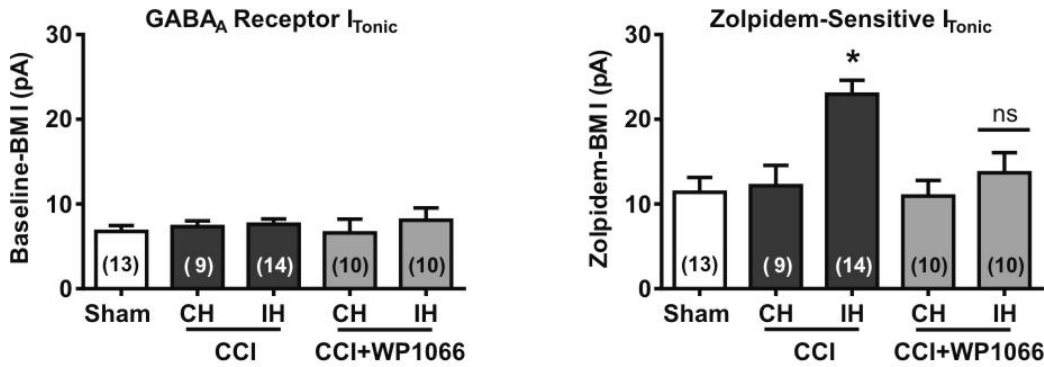
Figure 4. Graphical illustration of zolpidem effects on IPSCs 6-8 weeks post-injury.

Frequency - Experimental groups exhibited a significant main effect of sIPSC frequency under pre-zolpidem conditions [$F(4,49) = 6.17$, $p = 0.0004$]. Multiple comparisons revealed that DGCs ipsilateral to injury from CCI and CCI+WP1066 animals had significantly lower sIPSC frequencies compared to Sham controls (Tukey's, $p = 0.013$ and $p = 0.022$, respectively) or their contralateral cell counterparts controls (Tukey's, $p = 0.023$ and $p = 0.0028$, respectively). Ipsilateral cells from CCI versus CCI+WP1066 animals did not significantly differ in sIPSC frequency under these conditions (Tukey's, $p > 0.99$). A significant main effect of sIPSC frequency was also detected in the presence of zolpidem [$F(4,49) = 5.35$, $p = 0.0012$]. Multiple comparisons revealed that DGCs ipsilateral to injury from CCI or CCI+WP1066 animals had significantly lower sIPSC frequencies compared to contralateral cells from their respective groups (CCI IH versus CCI CH, Tukey's, $p = 0.024$) and (CCI+WP1066 IH versus CCI+WP1066 CH, Tukey's, $p = 0.036$).

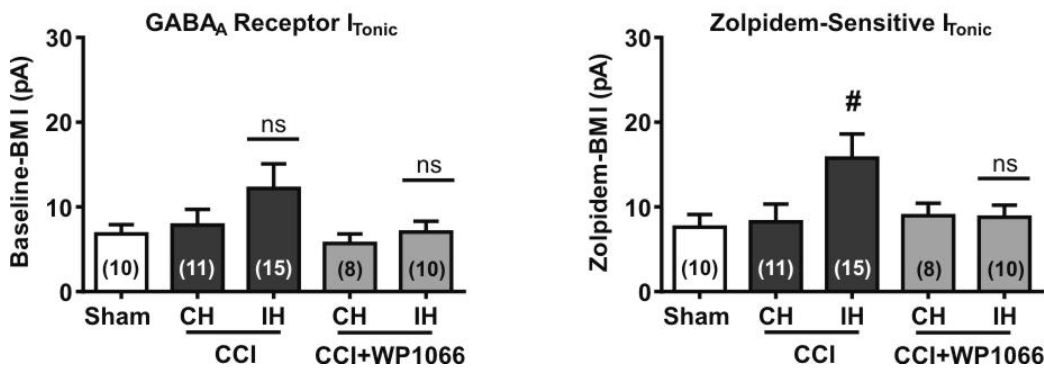
A statistical trend for each injured group (ipsilateral CCI and ipsilateral CCI+WP1066) was detected relative to Sham controls (Tukey's, $p = 0.060$ and $p = 0.069$, respectively). DGCs ipsilateral to injury from CCI versus CCI+WP1066 animals exhibited sIPSC that did not significantly differ from each-other (Tukey's, $p > 0.99$).

Amplitude - No significant main effect of condition was detected between groups in the amplitude of sIPSCs under zolpidem-free conditions [$F(4,49) = 0.29$, $p = 0.88$] or in the presence of zolpidem [$F(4,49) = 0.098$, $p = 0.98$]. **Decay Time Constant** - A significant main effect of condition was detected between groups for sIPSC decay time constant in the absence of zolpidem [$F(4,49) = 6.02$, $p = 0.005$]. Multiple comparisons revealed that DGCs ipsilateral to injury from CCI animals possessed a significantly greater sIPSC decay time constants compared to Sham controls (Tukey's, $p = 0.029$) or their contralateral cell counterpart controls (Tukey's, $p = 0.010$). Similarly, DGCs ipsilateral to injury from CCI+WP1066 animals possessed significantly greater sIPSC decay time constants compared to Sham controls (Tukey's, $p = 0.044$) or their contralateral cell counterpart controls (Tukey's, $p = 0.039$). A post hoc comparison of ipsilateral DGCs from CCI versus CCI+WP1066 animals detected no difference in sIPSC decay time constant for these groups in the presence (Tukey's, $p > 0.99$) or absence of zolpidem (Tukey's, $p > 0.99$). A comparison of zolpidem's percent augmentation of decay time constant revealed a significant main effect [$F(4,49) = 3.59$, $p = 0.012$]. Based on multiple comparisons, neither ipsilateral DGCs from CCI animals nor ipsilateral DGCs from CCI+WP1066 animals presented significant differences in zolpidem augmentation of decay time constants relative to Sham or contralateral controls. There were however, statistical trends for reduced zolpidem augmentation of decay time constants for ipsilateral cells from CCI and CCI+WP1066 conditions relative to Sham controls (Tukey's, $p = 0.076$ and $p = 0.10$, respectively). A post hoc comparison of ipsilateral DGCs from CCI versus CCI+WP1066 animals detected no difference in zolpidem augmentation of sIPSC decay time constant for these groups (Tukey's, $p > 0.99$).

I_{tonic} : 10-21 days



I_{tonic} : 6-10 weeks



Interestingly, this zolpidem augmentation of the Tonic GABA current was absent in ipsilateral DGCs from CCI+WP1066 animals thereby indicating an effect of WP1066 (versus Sham controls, Tukey's, $p = 0.92$ and versus contralateral CCI+WP1066, Tukey's, $p = 0.88$). **Bottom**, 6-10 weeks post-injury. Tonic GABA currents, in the absence of zolpidem, did not exhibit a significant main effect of condition between groups [$F(4,49) = 1.60$, $p = 0.19$]. A significant overall effect on Tonic GABA currents in the presence of zolpidem was detected [$F(4,49) = 2.74$, $p = 0.039$]. Subsequent multiple comparisons using Tukey's test identified no significant differences among individual conditions. However, statistical trends were observed for ipsilateral DGCs from CCI animals to possess larger zolpidem-sensitive Tonic GABA currents relative to Sham controls (Tukey's, $p = 0.069$) as well as their contralateral cell counterparts ($p = 0.095$). Ipsilateral cells from animals given CCI+WP1066 did not significantly differ from Sham controls (Tukey's, $p > 0.99$) or their contralateral cell counterparts ($p > 0.99$).

Figure 5. Graphical illustration of zolpidem on I_{tonic}. **Top**, 10-21 days post-injury. Tonic GABA currents, in the absence of zolpidem, did not exhibit a significant main effect of condition between groups [$F(4,51) = 0.34$, $p = 0.85$]. In contrast, a significant overall effect on Tonic GABA currents in the presence of zolpidem was detected [$F(4,51) = 7.63$, $p < 0.0001$]. Multiple comparisons revealed that DGCs ipsilateral to injury from CCI animals possessed a significantly larger zolpidem-sensitive Tonic GABA current compared to Sham controls (Tukey's, $p = 0.0003$) or their contralateral cell counterpart controls (Tukey's, $p = 0.0028$).

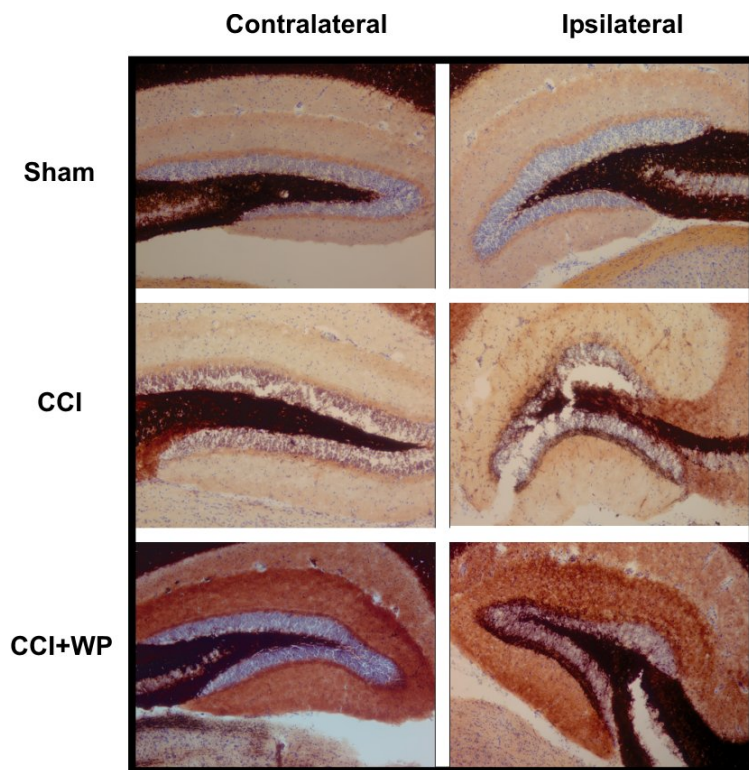


Figure 6. Timm staining in the dentate gyrus from sham control, CCI-injured, and CCI-injured+WP1066 treated mice. Images of the dentate gyrus contralateral (left images) and ipsilateral (right), directly under the ‘epicenter’ of the injury or skull opening in sham-operated controls, CCI-injured, or CCI-injured+WP1066-treated mice after ~8 weeks post-injury. Contralateral images are from approximately equivalent hippocampal levels contralateral to the skull opening. Mossy fiber sprouting into the inner molecular layer is not seen contralateral to the injury or skull opening. However, mossy fiber sprouting is evident ipsilateral to the injury in CCI-injured and CCI-injured+WP1066-treated mice. The degree of sprouting is similar for treated- and untreated CCI-injured mice and is similar to that seen previously to be associated with synaptic reorganization.

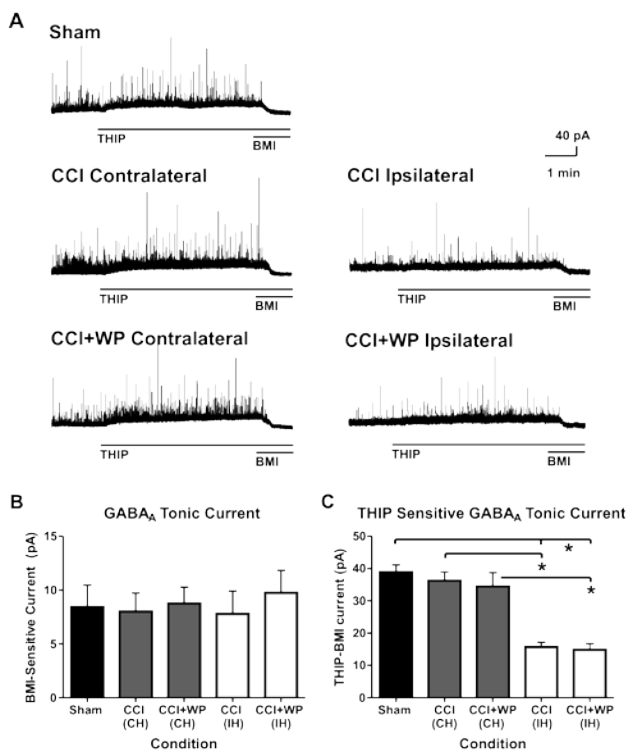


Figure 7. Reduced THIP-sensitive tonic GABA_A currents in dentate granule cells (DGCs) located ipsilateral to controlled cortical impact (CCI) during 6-13 weeks post-injury. A. Representative traces of tonic GABA_A currents in DGCs after sham injury (upper left; n=7), CCI injury (middle row; Contralateral: n=7, Ipsilateral: n=7) or CCI injury with acute WP1066 treatment (bottom row; Contralateral: n=5, Ipsilateral: n=7). DGCs were voltage clamped at 0 mV (near reversal potential of glutamatergic currents) and recorded in three phases: baseline, THIP (3 μM; Sigma, USA) and Bicuculline Methiodide (BMI; 30 μM; Tocris, USA). B. Group data of tonic GABA_A currents measured as the change in steady-state holding current values of baseline to BMI application (Baseline I_{Hold} – BMI I_{Hold}). C. Group data of THIP-sensitive tonic GABA_A currents measured as the change in steady-state holding current values of THIP application to bicuculline application (THIP I_{Hold} – BMI I_{Hold}). Given the recoding parameters here, an increase in tonic GABA_A

receptor-mediated currents using THIP produced an outward shift whereas blockade of GABA_A receptors with BMI produced an inward shift in the holding current. Data shown as mean ± SEM.

Table 1. IPSC frequency, amplitude and decay time constant in control, CCI-injured and CCI-injured+WP1066 treated mice; effects of THIP on IPSCs are also indicated.

sIPSCs	Sham	CCI				CCI+WP1066			
	Baseline	Contralateral Baseline	THIP	Ipsilateral Baseline	THIP	Contralateral Baseline	THIP	Ipsilateral Baseline	THIP
Frequency(Hz)	1.41±0.09	1.30±0.26	0.82±0.08	0.80±0.12*	0.68±0.08	1.33±0.34	0.74±0.35	0.72±0.09*	0.69±0.07
Peak Amplitude(pA)	20.36±1.92	20.89±1.88	22.11±3.05	22.85±3.02	20.93±2.24	21.72±2.58	23.34±3.36	18.71±1.57	21.22±1.78
10-90% Rise Time (ms)	2.23±0.10	2.29±0.18	2.24±0.44	2.47±0.11	2.53±0.18	2.30±0.13	2.33±0.09	2.45±0.18	2.42±0.12
Decay Time Constant (ms)	14.06±3.40	15.33±3.26	14.17±2.02	17.04±3.56	16.03±1.63	14.10±0.67	13.35±0.88	16.97±1.49	15.70±2.05

Synaptic responses were assessed at baseline and at steady-state during 4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridine-3-ol hydrochloride (THIP; 3 μ M) application. Sample sizes are sham injury (n=7), CCI injury (Contralateral: n=7, Ipsilateral: n=7) or CCI injury with acute WP1066 treatment (Contralateral: n=5, Ipsilateral: n=7). Data shown as mean \pm SEM. IPSC frequency was significantly reduced in CCI-injured mice relative to controls ($p<0.05$); WP1066 did not affect IPSC frequency.

Hall, E.D., Smith, B.N., Brooks-Kayal, A., and Soltesz, I. (2014) When ski helmets aren't enough: emerging therapies for TBI and post-traumatic epilepsy. *Winter Conf. Brain Res.*

Traumatic brain injury (TBI) is a major unmet medical need with an annual US incidence >1.5 million persons and a high incidence of lasting neurological deficits and sequelae including posttraumatic seizures (PTS) and posttraumatic epilepsy (PTE). TBI is highly relevant to alpine skiing and snowboarding injuries of which 17.6% are due to TBI. While use of ski helmets has cut down the incidence by as much as 60%, they do not eliminate the risk of TBI and PTE. At present, there are no FDA-approved neuroprotective treatments that improve post-TBI neurological recovery or that prevent PTS and PTE development in contrast to the increasing armamentarium of seizure suppressing compounds (aka anti-seizure drugs), which are not disease-modifying and can have negative effects on cognitive and sensorimotor recovery in TBI patients. This panel will discuss three newer approaches to the treatment of TBI that should attenuate PTE. Following a brief introduction to the epidemiology of TBI, PTS and PTE by organizer Ed Hall, Bret Smith (U. Kentucky) will present elegant studies on the pathophysiology of PTE development in mice subjected to controlled cortical impact TBI that leads to reorganization of circuits in the cortex and hippocampus such that GABA_A receptor (R)-mediated inhibition is decreased while recurrent excitatory circuits are increased. Amy Brooks-Kayal (U. Colorado) will then discuss her recent work on the involvement of the JAK/STAT pathway in the TBI-induced down-regulation of GABA_A R-dependent synaptic inhibition and the utility of JAK/STAT inhibitors for prevention of PTE development. Next, Ivan Soltesz (U. California-Irvine) will discuss the therapeutic potential of optogenetics for treatment of PTE. Finally, Ed Hall (U. Kentucky) will discuss the role of free radical-induced lipid peroxidation in post-traumatic brain damage including PTE development and protective efficacy of newer brain-penetrable antioxidant compounds.